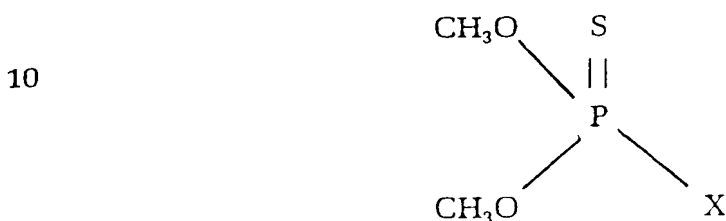
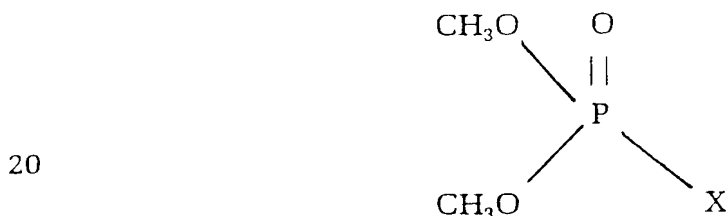


## MALATHION CARBOXYLESTERASE

This invention relates to an enzyme (and the nucleic acid sequences encoding this enzyme), termed malathion carboxylesterase (MCE) which is able to efficiently hydrolyse a specific class of organophosphate (OP) insecticides which have the general structures:



15 (eg. malathion, phenthoate)



(eg. malaoxon, phenthoate oxon)  
where X contains one or more carboxylester groups for thion type organophosphates but is unconstrained for oxon type organophosphates.

Residues of organophosphate insecticides are undesirable contaminants of the environment and a range of commodities. Areas of particular sensitivity include contamination of domestic water supplies and soil, residues above permissible levels in various food and fibre exports and contamination of domestic pets. Bioremediation strategies are therefore required for eliminating or reducing these insecticide residues. One proposed strategy involves the use of enzymes capable of immobilising or degrading the insecticide residues. Such enzymes may be employed, for example, in bioreactors through which contaminated water could be passed; in production animal dips to reduce problems with contaminated pasture and run off into water supplies; or in washing solutions after post harvest

disinfestation of fruit, vegetables or animal products to reduce residue levels and withholding times. Suitable enzymes for degrading pesticide residues include esterases. It is desirable that the esterases be relatively specific and hydrolyse the pesticide residues at a rapid rate.

5       The MCE enzyme has been purified from different malathion resistant strains of *L. cuprina*, RM and der-L (Whyard S., Russell R.J. and Walker V.K., Biochemical Genetics 32: 9, 1994; Whyard S. and Walker V.K., Pesticide Biochemistry and Physiology 50: 198, 1994). It is a 60.5 kDa monomer with a  $K_m$  for malathion of  $11.0 \pm 0.4 \mu M$  and a  $V_{max}$  of  $775 \pm 28$  nmol malathion/min/mg. It also has a high turnover rate for malathion ( $k_{cat} = 46 \text{ min}^{-1}$ ).

10       In order to enable the production of useful amounts of the MCE enzyme the present inventors sought to clone the putative MCE gene from a malathion resistant strain of *L. cuprina*(RM-8) using PCR and cloning techniques.

15       The MCE gene in *L. cuprina* has been mapped using classical genetic techniques to a position within 0.7 map units from the E3 gene on chromosome 4. The likely homologue of MCE in *Drosophila melanogaster*, *Mce*, has been mapped to the right arm of chromosome 3 in the vicinity of the genes encoding the major  $\alpha$ -carboxylesterase, EST 9, and the orthologue of *L. cuprina* E3, EST23 (Spackman M.E., Oakeshott J.G., Smyth K-A., Medveczky K.M., and Russell R.J., Biochemical Genetics, 32: 39, 1994).

20       In order to clone the MCE gene from *L. cuprina*, it was decided to use the wealth of molecular genetic techniques available for *D. melanogaster* to clone the MCE homologue and use these clones as probes to isolate the *L. cuprina* genes themselves.

25       In summary, five esterase amplicons were isolated from *L. cuprina* genomic and cDNA. Four of the five *L. cuprina* amplicons obtained by PCR using cluster specific primers were designated Lc $\alpha$ E7, Lc $\alpha$ E8, Lc $\alpha$ E9 and Lc $\alpha$ E10 on the basis of homology to the corresponding *Drosophila* genes. The fifth, Lc#53, could not be assigned with any confidence on the basis of similarity to any of the *Drosophila* genes.

30       MCE specific activity is highest in the adult head, rather than the thorax or abdomen (Smyth,K-M., Walker,V.K., Russell,R.J. and Oakeshott,J.G. Pesticide Biochemistry and Physiology. 54:48, 1996). On this basis, Lc $\alpha$ E7, Lc $\alpha$ E8 and Lc $\alpha$ E10 were all MCE candidates. Previous

physiological studies of Parker, A.P., Russell, R.J., Delves, A.C. and Oakeshott, J.G. (Pesticide Biochemistry and Physiology 41:305, 1991) have shown that the E3 (Lc $\alpha$ E7) enzyme is present in the adult head. Moreover, the Lc $\alpha$ E8 and Lc $\alpha$ E10 genes are also expressed in the head since PCR using  
5 cluster-specific primers were able to amplify these genes from a head cDNA library. PCR failed to detect Lc $\alpha$ E9 and Lc#53 in either larval fat body or adult head cDNA and Northern analysis of the *D. melanogaster*  $\alpha$ E9 homologue indicated that this gene was only expressed in embryos. Therefore both Lc $\alpha$ E9 and Lc#53 were discounted as candidates for the  
10 genes encoding E3 and MCE.

The Lc $\alpha$ E8 and Lc $\alpha$ E10 genes were initially chosen as prime MCE candidates on the basis of this distribution and due to the fact that it was known that Lc $\alpha$ E7 encodes the E3 enzyme involved in diazinon/parathion OP resistance in *L. cuprina* (PCT/AU 95/00016: "Enzyme based  
15 bioremediation") and it was thought that malathion resistance and diazinon/parathion resistance were encoded by separate genes.

The present inventors have made the surprising finding that it is a variant of Lc $\alpha$ E7 which encodes the MCE enzyme. This gene has been expressed *in vitro* and the product shown to have MCE activity. The  
20 expressed product can be formulated for use in degrading environmental carboxylester or dimethyl general OPs.

Accordingly, in a first aspect, the present invention consists in an isolated DNA molecule encoding an enzyme capable of hydrolysing at least one organophosphate selected from the group consisting of carboxylester  
25 organophosphates and dimethyl-oxon organophosphates, the DNA molecule comprising a nucleotide sequence having at least 60%, preferably at least 80% and more preferably at least 95% homology with Lc $\alpha$ E7, in which the protein encoded by the DNA molecule differs from E3 at least in the substitution of Trp at position 251 with an amino acid selected from the  
30 group consisting of Leu, Ser, Ala, Ile, Val, Thr, Cys, Met and Gly.

In a preferred embodiment the present invention the isolated DNA molecule has a sequence as shown in Figure 1 or a sequence which hybridises thereto with the proviso that the protein encoded by the DNA molecule differs from E3 at least in the substitution of Trp at position 251  
35 with an amino acid selected from the group consisting of Leu, Ser, Ala, Ile, Val, Thr, Cys, Met and Gly.

In a preferred embodiment of the present invention the Trp at position 251 is substituted with Leu or Ser.

As is stated above the present invention includes nucleic acid molecules which hybridise to the sequence shown in Figure 1. Preferably such hybridisation occurs at, or between, low and high stringency conditions. In general terms, low stringency conditions can be defined as 3xSSC at about ambient temperature to 65°C, and high stringency conditions as 0.1xSSC at about 65°C. SSC is the abbreviation of a buffer of 0.15M NaCl, 0.015M trisodium citrate. 3xSSC is three times as strong as SSC and so on.

10 In a second aspect the present invention consists in an isolated DNA molecule, the DNA molecule encoding a polypeptide having the amino acid sequence of RM-8Con shown in Figure 1 or the amino acid sequence of MdαE7 shown in Figure 3 in which Trp at position 251 is replaced with Ser.

15 Homologues of the MCE encoding sequence may also be present in the genome of other insects, and particularly other species of Diptera. Thus, it is to be understood that the invention also extends to these homologues. An example of this is provided by the results set out hereunder regarding *Musca* MCE.

20 The isolated DNA molecules of the present invention may be cloned into a suitable expression vector and subsequently transfected into a prokaryotic or eukaryotic host cell for expression of the enzyme. A particularly suitable system involves baculovirus vectors and an insect cell line.

25 In a third aspect the present invention consists in a method of producing an enzyme capable of hydrolysing at least one organophosphate selected from the group consisting of carboxylester organophosphates and dimethyl-oxon organophosphates, or an enzymatically active portion thereof, the method comprising transforming a host cell with the DNA molecule of the first aspect of the present invention operatively linked to a control  
30 sequence, culturing the transformed cell under conditions which allow expression of the DNA sequence and recovering the produced enzyme, or enzymatically active portion thereof.

35 It is also envisaged that as an alternative to using the enzyme *per se* as a bioremediation agent the bioremediation agent may be an organism transformed with the DNA encoding the enzyme. In such an arrangement

the organism, transformed such that it expresses the enzyme, would be used as the bioremediation agent.

The invention further relates to methods for eliminating or reducing the concentration of carboxylester or dimethyl-oxon-type organophosphate insecticides residues in a contaminated sample or substance, involving the  
5 use of an esterase encoded by an isolated DNA molecule according to the present invention.

In order that the nature of the present invention may be more clearly understood preferred forms will now be described with reference to the  
10 following examples and Figures in which:

Figure 1 shows <sup>Fig. 1A</sup> multiple nucleotide alignment of the three malathion-resistant clones (RM8A-C) and their consensus (RM8con) with the reference susceptible clone (Lc743) of Lc $\alpha$ E7 (E3). Dots indicate identity with the Lc743 susceptible clone. Below the ruler is the aligned nucleotide  
15 sequence and above is the inferred amino acid sequence of Lc743 with the one replacement found in Lc7RM8con indicated in bold text immediately below. Nucleotides are numbered from the predicted start of translation and amino acids from the predicted start methionine. Lc743 5' and Lc743 3' primer sequences are underlined.

Figure 2 shows <sup>Fig. 2A and 2B</sup> amino acid alignment of the inferred MdaE7 protein from the Rutgers strain of *Musca domestica* compared to the Lc $\alpha$ E7 (E3) protein from the *Lucilia cuprina* Lc743 clone (PCT/AU95/00016 "Enzyme Based Bioremediation"). Sequence comparison shows a 75% identity and  
20 86% similarity between the same length, 570 residue proteins. Arrow indicates the conserved tryptophan residue at position 251 of the alignment.

Figure 3 shows <sup>Fig. 3A and 3B</sup> the 1710bp nucleotide coding sequence of the Rutgers strain MdaE7 gene. Also shown is the inferred 570 protein sequence.

Figure 4 shows amino acid alignment of the PCR Ankara strain  
30 MdaE7 amplicon and the corresponding region of the RM-8 malathion resistant Lc $\alpha$ E7 protein. The structural mutations conferring malathion resistance (serine for MdaE7 and Leucine for LcaE7) are indicated by arrow at residue position 251.

## CLONING AND SEQUENCING OF THE MCE GENE FROM A MALATHION RESISTANT STRAIN OF LUCILIA CUPRINA

Two types of change in carboxylesterase activity have been  
5 associated with resistance to OP insecticides in the higher Diptera. One type  
of change results in resistance to OPs like diazinon and parathion, while the  
other results in resistance to OPs like malathion, with one or more  
carboxylester groups in addition to the phosphotriester moiety that defines it  
as an OP (see above).

10 The two types of change were first described among OP resistant  
strains of *Musca domestica*. In both types an increased degradation of OPs  
was associated with reduced ali-esterase activity, where "ali-esterase" refers  
to enzymes which are major contributors to the hydrolysis of the  
carboxylester, methyl butyrate, or similar molecules (Oppenoorth, F.J.,  
15 Entomology Experimental and Applied, 2: 304, 1959; Oppenoorth F.J. and  
van Asperen, K., Entomology Experimental and Applied 4: 311, 1961). This  
led to the formulation of the "mutant ali-esterase hypothesis", which  
proposes that each type of resistance is due to a mutation in a specific  
carboxylesterase that simultaneously enables it to hydrolyse the  
20 phosphoester linkages common to the oxon form of all OPs and decreases its  
activity toward certain carboxylester substrates (Oppenoorth, F.J. and van  
Asperen, K., Science 132: 298, 1960).

Both types of change yielded resistance factors for diverse OPs  
(except malathion) in the range of about 2-30 fold (Bell, J.D. and Busvine,  
25 J.R., Entomology Experimental and Applied, 10: 263, 1967). However, the  
malathion resistant *M. domestica* strains also showed exceptionally high  
resistance to malathion (generally >100 fold). This high resistance was  
associated with cleavage of the carboxylester linkages in malathion (ie MCE  
activity) in addition to the hydrolysis of the phosphoester linkage (OP  
30 hydrolase activity). MCE activity accounted for the major breakdown  
products *in vivo* and *in vitro* (Townsend, M.G. and Busvine, J.R., Entomology  
Experimental and Applied 12: 243, 1969).

No recombination between the two types of resistance or between  
them and ali-esterase activity was observed among the *M. domestica* strains  
35 (Nguy, V.D. and Busvine, J.R., World Health Organisation 22: 531, 1960).  
This suggests that while they are clearly distinct in respect of OP hydrolase

and MCE activities, the two types of resistance might nevertheless be allelic changes to the same carboxylesterase/ali-esterase gene/enzyme system (Oppenoorth, F. J. and Welling, W., in Insecticide Biochemistry and Pharmacology, Wilkinson, C.F. ed., Plenum Press, New York and London, pp. 507-551, 1976).

A malathion resistance phenotype has also been described in the blowfly, *Chrysomya putoria*, which parallels the malathion resistance phenotype of *M. domestica* in that it is associated with high MCE and low ali-esterase activities (Busvine, J.R., Bell, J.D. and Guneidy, A.M., Bulletin of Entomological Research 54: 589, 1963; Bell, J.D. and Busvine, J.R., Entomology Experimental and Applied 10: 263, 1967; Townsend, M.G. and Busvine, J.R., Entomology Experimental and Applied 12: 243, 1969). Further evidence for the similarity of the malathion resistance phenotypes in the two species is indicated by the spectrum of OP compounds which synergise malathion. Specifically, among a series of symmetrical trisubstituted phosphorus compounds, the best synergists (eg triphenylphosphate) were common to both species (Bell, J.D. and Busvine, J.R., Entomology Experimental and Applied 10: 263, 1967). However, little is known of diazinon/parathion type resistance in *C. putoria*.

The mutant ali-esterase hypothesis has also been invoked to explain diazinon/parathion resistance in *L. cuprina*, because these flies hydrolyse paraoxon more rapidly than susceptible flies (Hughes, P.B. and Devonshire, A.L., Pesticide Biochemistry and Physiology 18:289, 1982) and resistance is associated with reduced carboxylesterase activity. In this case the esterase isozyme E3 from resistant flies is not detected ("non-staining") after polyacrylamide gel electrophoresis (PAGE; Hughes, P.B. and Raftos, D.A., Bulletin of Entomological Research 75: 535, 1985). Evidence for a causal connection between the E3 change and resistance was obtained by EMS mutagenesis of an E3 staining, OP susceptible strain of *L. cuprina* and selection for OP resistant mutants; all resistant mutants recovered had the E3 non-staining PAGE phenotype (McKenzie, J.A., Parker, A.G. and Yen, J.L., Genetics 130: 613, 1992).

Like malathion resistant strains of *M. domestica*, strains of *L. cuprina* that are resistant to malathion exhibit very high resistance factors towards malathion and enhanced MCE activity. Also in common with *M. domestica*, malathion resistant *L. cuprina* generally do not exhibit

diazinon/parathion resistance, and *vice versa*. However, one difference from the situation in *M. domestica* is that the loci encoding the two resistance phenotypes appeared in some experiments to be genetically separable, albeit closely linked (Smyth, K-A., Russell, R.J. and Oakeshott, J.G., Biochemical Genetics 32: 437, 1994; Smyth, K-A., Walker, V.K., Russell, R.J. and Oakeshott, J.G., Pesticide Biochemistry and Physiology 54: 48, 1996)..

An esterase gene cluster containing genes involved in OP resistance has been isolated from *L. cuprina* (Newcomb, R.D., East, P.D., Russell, R.J. and Oakeshott, J.G., Insect Molecular Biology 5: 211, 1996). One of these genes, *LcaE7*, encodes esterase E3 (Newcomb, R.D., Campbell, P.M., Russell, R.J. and Oakeshott, J.G. Insect Biochemistry and Molecular Biology, in press), a structural mutation in the active site of which confers diazinon/parathion resistance on *L. cuprina*. These data are described in a previous patent application PCT/AU 95/00016: "Enzyme based bioremediation", the disclosure of which is incorporated herein by reference.

Below we describe the cloning and sequencing of the *LcaE7* gene from a malathion resistant strain of *L. cuprina*. We present molecular genetic evidence that this allele of esterase E3 is the MCE gene responsible for malathion resistance in *L. cuprina*.

a) Cloning the malathion resistant allele of *LcaE7*

An RT-PCR (reverse transcriptase - PCR) approach was used to clone a cDNA allele of *LcaE7* from a malathion resistant strain of *L. cuprina* (RM-8) which is homozygous for the fourth chromosome.

Adults from the RM-8 strain were aged for three days before collection and stored at -70° C. RNA was prepared using a modified protocol of Chigwin *et al.* (Chigwin, J.M., Przybyla, A.E., MacDonald, R. J. & Rutter, W.J., 1979, Biochemistry 18, 5294). About 100 adults were thoroughly homogenised in 15ml of solution D (4M guanidinium thiocyanate, 25mM sodium citrate, pH 7.0, 0.5% sarkosyl, 0.1M  $\beta$ -mercaptoethanol) using a Sorvall Omnimix blender. The resulting homogenate was filtered through glasswool and 6 ml layered on top of 5ml of 4.8M CsCl, made up in 10mM Na- EDTA, pH 8, in an SW41 ultracentrifuge tube. These were spun at



35,000 rpm in an SW41 rotor for 16hr at 15° C. The supernatant was removed and the RNA pellet resuspended in 400 µl of DEPC-treated H<sub>2</sub>O. The RNA was precipitated by the addition of 800 µl of ethanol and 10µl of 4M NaCl and stored under ethanol at -20°C. Before use the RNA pellet was washed in 75% ethanol and air dried before resuspension in DEPC-treated H<sub>2</sub>O.

PolyA<sup>+</sup> RNA was prepared from 500 µg of total RNA using affinity chromatography on oligo-dT cellulose (Pharmacia; Sambrook, J., Fritsch, E.F., and Maniatis, T., 1989, Molecular Cloning: A Laboratory Manual, 2nd Edition, Cold Spring Harbor Laboratory Press, USA). The resulting mRNA (1-5µg) was again precipitated, washed and resuspended in 20µl of DEPC-treated H<sub>2</sub>O. Oligo-dT primed cDNA was made from 1µg of mRNA using reverse transcriptase (Superscript II, BRL) as per the manufacturers instructions in a 20µl volume reaction. 200ng of cDNA was used as template in each of two PCR reactions using primers designed from the 5' (Lc743 5': 5' atgaatttcaacgtagtttgatgga 3') and complementary 3' (Lc743 3' : 5' ctaaaataaatctctatgttttcaaac 3') ends of the coding region of the LcαE7 gene. Reactions used Taq DNA polymerase (BRL) and contained 100 pmoles of each primer, 0.2 mM of each dNTP, 10mM Tris-HCl, pH 8.3, 50mM KCl, 0.002% Tween 20 (v/v), 1.5 mM MgCl<sub>2</sub>, and 200 ng of template. Two drops of mineral oil were layered over each 50µl reaction. Six units of Taq enzyme was added after a 5 minute "hot start" at 97°C and was followed by 40 cycles of 35 seconds at 97°C, 1 minute at 60°C and 2 minutes at 72°C. A final cycle of 72°C for 8 minutes was included. The 1.7 kb major product was gel purified and cloned into the EcoRV cleavage site of the pBSK<sup>-</sup> (Stratagene) or pGEM-T (Promega) plasmid vectors using conventional cloning techniques (Sambrook, J., Fritsch, E.F., and Maniatis, T., 1989, Molecular Cloning: A Laboratory Manual, 2nd Edition, Cold Spring Harbor Laboratory Press, USA).

### 30 b) Sequencing the malathion resistant allele of LcαE7

#### *Methods:*

Three clones were chosen for sequencing (RM8-A to -C), all of which were derived from independent PCR reactions. A set of twelve

21-mer sequencing primers (sequence shown below) were designed from the existing Lc $\alpha$ E7 sequence:

Table 1

5

primer seq (5' - 3')	primer name	5' position in Lc743 sequence (Figure 1)
ggatgggtgtgcgtgattgttg	7F1	246
aaaaggatgtggtgttgatta	7F2	464
actaatgtcgggtaatgctat	7F3	723
cactatgatgggtaacacttc	7F4	1026
tgttacaggagaaacaccaac	7F5	1203
agaatcgcgtgaatacaaaac	7F6	1467
acgggtataccctcaaaactgt	7R1	187
tcccaaacgatattgtatgtt	7R2	504
acatcatgtagtgggtagaag	7R3	685
ccgaggatgttgggtaagac	7R4	990
tatcagctgttgggttttctc	7R5	1231
acgcgattctttaggcatacg	7R6	1476

These were used in dye-terminator sequencing reactions (ABI) conducted following manufacturer's instructions in 25 $\mu$ l capillary tubes in a Corbett Research capillary thermal cycler, except that 50pmoles of primer was used per reaction, a "hot start" of 96°C for 3 minutes was included and 30 cycles were completed for each sequencing reaction. Dye primer reactions were also conducted on all clones using the ABI M13 forward and reverse primers as per ABI protocols. Sequencing reactions were resolved by electrophoresis on an ABI 370A automatic sequencing machine as per the manufacturer's instructions. This resulted in both strands being sequenced entirely.

### Results:

20

Figure 1 shows a nucleotide and amino acid alignment of the three resistant clones (RM8 A-C) compared with the reference susceptible clone

(Lc743) of Lc $\alpha$ E7. A consensus sequence of the malathion-resistant Lc $\alpha$ E7 allele was determined (RM-8con). Differences between resistant clones were assumed to be errors incorporated by the Taq polymerase.

5 Comparison of the susceptible sequence (Lc743) with that of the malathion-resistant RM-8 consensus sequence (RM-8con) identified only one replacement site difference, a Trp to Leu substitution at amino acid position 251 (nucleotide position 752). The homologous amino acid was highlighted on a three-dimensional model of *T. californica* AChE, revealing that the Leu mutation was situated at the base of the active site gorge, 6.5 Angstroms  
10 from the active site Ser.

c) Sequencing the region surrounding nucleotide 752 from various Lc $\alpha$ E7 alleles

15 An esterase structural mutation conferring malathion resistance would be expected to occur in the active site region of the molecule. The Trp to Leu mutation at nucleotide position 752 in Lc $\alpha$ E7 is therefore an excellent candidate for the malathion resistance mutation.

20 The inventors have established a total of 14 strains of *L. cuprina* which are homozygous for chromosome IV and of known malathion resistance status. These lines fall into seven classes on the basis of an RFLP analysis of genomic DNA using the Lc $\alpha$ E7 gene as a probe. Nucleotide position 752 was therefore sampled over the entire range of classes.

25 **Methods:**

The complete cDNA sequence of the Lc $\alpha$ E7 alleles from strains representing several of the classes are available. For example, the sequence of Lc $\alpha$ E7 from RM-8 is shown in Figure 1<sup>A, B, C, D</sup>. Moreover, the Lc $\alpha$ E7 cDNA  
30 sequences from strains LS2 and Llandillo 103, which represent two more classes, are described in patent application PCT/AU 95/00016 ("Enzyme based bioremediation"). The complete Lc $\alpha$ E7 cDNA sequence of the Gunning 107 strain, representing a fourth class, is described in J. Trott, B.Agr.Sc Thesis, 1995.

35 To obtain the sequence of Lc $\alpha$ E7 in the region of nucleotide 752 in strains LBB101, Llandillo 104 and Hampton Hill 6.2, representing the

remaining three classes, a PCR approach was taken. Genomic DNA was prepared from either eggs using the method of Davis, L.G., Dibner, M.D., and Batley, J.F., (1986. *Basis Methods in Molecular Biology*, Elsevier Science Publ. Co., New York, Section 5.3), or from adult flies using a C-TAB method (Crozier, Y.C., Koulianos, S. & Crozier, R.H., 1991, *Experientia* 47, 9668-969). 1µg samples were then used as templates in PCR reactions using 100pmoles of the primers 7F1 and 7R4. Also included in the reactions were 0.2mM of each dNTP, 10mM Tris-HCl, pH 8.3, 50mM KCl, 1.5mM MgCl<sub>2</sub>. Two drops of mineral oil were layered over each 50µl reaction. 2.5 units of Taq polymerase was added after a 'hot start' of 97°C for 3 minutes while an annealing temperature of 55° was maintained. An initial extension at 72°C was held for 2 minutes. This was followed by 34 rounds of 97°C for 35 seconds, 55°C for 1 minute and 72°C for 1 minute. A final extension of 72°C for 9 minutes was included. A single product of about 1kb was produced. This was purified for sequencing using QIAquick spin columns (Qiagen), following manufacturer's instructions. 500 ng of template was used in dye-terminator sequencing reactions using the 7F7 (5': 5'tgctgcctctaccactacat 3') and 7R7 (3': 5' cctgtggccttgctttcataa 3') primers as described above.

### Results:

Of the seven classes assayed, all five malathion-susceptible strains (LS2, LBB101, Llandillo 104, Gunning 107 and Llandillo 103) possess a G at nucleotide position 752, whereas both malathion-resistant strains (Hampton Hill 6.2 and RM-8) possess a T at this position, resulting in a Trp to Leu substitution at amino acid position 251 (Table 2). The presence of the same structural mutation in two malathion resistant strains with different fourth chromosomes strongly suggests that the mutation is responsible for resistance.

Table 2

Strain	Malathion resistance status	Class	Residue at amino acid position 251 <sup>a</sup>
LS2	Susceptible	A	Trp
LBB101	Susceptible	C	Trp
Llandillo 104	Susceptible	B	Trp
RM-8	Resistant	E	Leu
Hampton Hill 6.2	Resistant	F	Leu
Llandillo 103	Susceptible	D	Trp
Gunning 107	Susceptible	G	Trp

<sup>a</sup>Amino acid at position 251 corresponds to nucleotide position 752 in Figure 1.

5

d) Cloning and sequencing the orthologous  $\alpha$ E7 gene from a malathion resistant strain of *Musca domestica*

As described above, the diazinon/parathion and malathion esterase-mediated OP resistance types exhibit many striking parallels between *L. cuprina*, *M. domestica* and *C. putoria*, and are probably caused by functionally equivalent mutations in orthologous genes. The orthologous gene was therefore cloned from the housefly, *M. domestica*, and the region surrounding nucleotide 752 examined for the presence of the malathion resistance mutation in a malathion resistant *Musca* strain.

15

**PCR reactions:**

Consensus generic  $\alpha$ -esterase primers were designed to the conserved regions of the multiple amino acid alignments of *D. melanogaster* (Robin, C. Russell, R.J., Medveczky, K.M. and Oakeshott, R.J., Journal of Molecular Evolution 43: 241, 1996) and *L. cuprina* (Newcomb, R.D., Campbell, P.M., Russell, R.J. and Oakeshott, J.G. Insect Biochemistry and Molecular Biology, in press)  $\alpha$ -esterase genes, and used in a PCR amplification experiment for the recovery of homologous  $\alpha$ E7 gene sequence from *M. domestica*.

25

Genomic DNA was prepared using the Lifton method (Bender, W., Spierer, P. and Hogness, D.S., Journal of Molecular Biology 168: 17, 1989) from adult females of the Rutgers OP resistant housefly strain (Plapp, F.W. Jr., Tate, L.G. and Hodgson, E. 1976. Pestic. Biochem. Physiol. 6:175-182).

- 5 Rutgers strain genomic DNA was used as the template in a 50 $\mu$ l amplification reaction:

Table 3

	Final concentration/amount
Template DNA	100ng
primer Md1	50pmoles
primer Md2	50pmoles
Buffer	10mM Tris-HCl (pH8.3), 1.5mM MgCl <sub>2</sub> , 50mM KCl
dNTP's	0.25mM (dATP,dCTP,dTTP,dGTP)
Taq polymerase	1 unit
Total volume	50 $\mu$ l

- 10 Primers:

Md1 (35mer)

5' TTCGAGGGIATICCITAYGCIMARCCICCI BTNGG 3' (Seq. 10-130)  
corresponding to residues 58-69 in *L.cuprina*  $\alpha$ E7

Md2 (32mer)

- 15 5' ACYTGRTCYTTIARICCGICRTTICCI GGNAC 3' (Seq. 11-130)  
corresponding to residues 92-82 in *L. cuprina*  $\alpha$ E7

Note: IUB codes used for mixed positions; I = inosine.

- 20 PCR conditions over 38 cycles:

95°C 3' 1 cycle

80°C Hold (addition of 1 unit Taq polymerase)

95°C 1' 50°C 1' 72°C 1' 1 cycle

95°C 1' 55°C 1' 72°C 1' 35 cycles

- 25 95°C 1' 55°C 1' 72°C 4' 1 cycle

### ***Cloning and sequencing PCR amplicons:***

The 540bp major product was eluted from an agarose gel, purified using QIAGEN QIAquick PCR purification kit and cloned into the pGEM-T plasmid vector (PROMEGA) using standard techniques (Sambrook, J., Fritsch, E.F. and Maniatis, T., *Molecular Cloning: A Laboratory Manual*, Second Edition, Cold Spring Harbor Laboratory Press, USA, 1989). The ends of the cloned insert were sequenced using commercially available T7 and SP6 primers and TaqFS dye-terminator technology (ABI) on the Applied Biosystems Model 370A automated DNA sequencer. Translated amino acid sequences were aligned to predicted  $\alpha$ -esterase protein sequences using PILEUP from the GCG computer package (Devereux, J., *The GCG sequence analysis software package Version 6.0*. Genetics Computer Group, University of Wisconsin Biotechnology Center, 1710 University Avenue, Madison, Wisconsin, USA, 1989); all proved to be homologous to the sequences of known  $\alpha$ -esterase genes from *D. melanogaster* and *L. cuprina*. The cloned 534bp amplicon showed 76% identity over the equivalent 135 amino acids of the *L. cuprina*  $\alpha E7$  predicted protein sequence.

### ***Isolation of the complete $\alpha E7$ gene from M. Domestica:***

A  $\lambda$ DASH (Stratagene) genomic library of the Rutgers strain of *M. domestica* (Koener, J.F., Carino, F.A. and Feyereisen, R., *Insect Biochemistry and Molecular Biology* 23:439, 1993) was screened for a full-length genomic clone of  $\alpha E7$ . Approximately 300,000 plaques were probed with the  $^{32}\text{P}$  labelled 534bp amplicon described above. Library screening using conventional techniques (Sambrook, J., Fritsch, E.F. and Maniatis, T., *Molecular Cloning: A Laboratory Manual*, Second Edition, Cold Spring Harbor Laboratory Press, USA, 1989) was performed at high stringency (50% formamide, 5X SSC, 3X Denhardt's, 0.5% SDS and 10 $\mu\text{g/ml}$  salmon sperm DNA at 45°C) and included a final high stringency wash (0.1% SSC, 0.1% SDS at 65°C). Restriction mapping indicated that a single  $\lambda$ DASH clone with a 17.5kb genomic insert contained the  $\alpha E7$  gene. A 4.5kb HindIII fragment was subcloned into the pBSK<sup>+</sup> vector (Stratagene) and characterised using dye-terminator automatic sequencing technology, as described above. A set

a thirteen sequencing primers were designed and used to interpret the full length genomic sequence:

Table 4

primer name	5' - 3' primer sequence	size	5' position in MdaE7 coding sequence (Figure 3)
T7	end sequencing of 4.5kb pBSK <sup>+</sup> clone		polycloning site
T3	end sequencing of 4.5kb pBSK <sup>+</sup> clone		polycloning site
AE7.1	TTTGGTCCCGACTACTTTATGA	22mer	442 (See FIG. 1)
AE7.2	TGCCACTTATGAAATCTGTCTGTA	24mer	310 (See FIG. 1)
AE7.3	TACATGATGATAACCGAACAGACC	24mer	676 (See FIG. 1)
AE7.4	TCGATTATTTGGGTTTCATTTGT	23mer	107 (See FIG. 1)
AE7.5	ACAGACAGATTTTCATAAGTGG	21mer	288 (See FIG. 1)
AE7.6	TTTGCAATTCCTTCGGGTGTCA	21mer	913 (See FIG. 1)
AE7.7	ATTCGATACCCACATTGATAG	21mer	1016 (See FIG. 1)
AE7.8	GGCACTCCCATTTATTTGTAT	21mer	1312 (See FIG. 1)
AE7.10	ATGACTTTTCTGAAGCAATTCAT	23mer	1 (See FIG. 1)
AE7.11	AAACAATTCCTTCTTTTTATCGA	23mer	1710 (See FIG. 1)
AE7.12	GGCATGGAAAACCTCACCTGG	21mer	1558 (See FIG. 1)

5

The predicted coding sequence of 1710bp or 570 amino acids showed a very high 75% identity and 85% similarity to the equivalent full length 570 residues of the  $\alpha$ E7 protein from *L. cuprina* (Figures 2 and 3). Southern hybridisation analysis as per standard methods (Sambrook, J., Fritsch, E.F. and Maniatis, T., Molecular Cloning: A Laboratory Manual, Second Edition, Cold Spring Harbor Laboratory Press, USA, 1989) of genomic DNA from the Rutgers strain using the 534bp  $\alpha$ E7 PCR amplicon as a probe and EcoRI, Hind III, Sal I digested DNA, showed a single 4.5kb hybridising band for the HindIII digest, a single 6.0kb SalI band and two EcoRI bands of 1.5kb and 0.8kb. The analysis confirms the restriction pattern interpreted from the sequencing and mapping data of the lambda genomic clone. No other aberrant hybridisation patterns occurred indicating a high probability that  $\alpha$ E7 exists as a single copy gene.

10

15



**Characterisation of a putative  $\alpha E7$  malathion resistant allele of *M. domestic*:**

Genomic DNA extracted (Bender, W., Spierer, P. and Hogness, D.S.,  
5 Journal of Molecular Biology 168: 17, 1989) from single adult female flies of  
the highly malathion resistant Ankara strain (Sisli, M.N., Bosgelmez, A.,  
Kocak, O., and Porsuk, H. 1983. Mikrobiyol Bul. 17:49-46) was used for  
sequence characterisation of a putative malathion resistance allele. A series  
10 of PCR reactions were performed using single fly genomic DNA for the  
characterisation of allelic variants of the  $\alpha E7$  gene. PCR amplification using  
conditions described above, with the specific housefly AE7.5 and AE7.6  
primer pair, produced single amplicons of approximately 760bp. This  
amplicon encompasses the highly conserved region involved in the catalytic  
15 site of the enzyme, coding for residues 96-304 of the translated sequence,  
including the site of the Trp to Leu mutation at amino acid residue 251  
associated with malathion resistance in *L. cuprina* (Figure 4). Cloning and  
sequencing of PCR amplicons (described earlier) from nine individual flies  
showed that the Ankara strain segregates for two allelic variants of the  $\alpha E7$   
20 gene: one has a Trp residue at amino acid position 251, whereas the other  
has a Ser at this same position. This replacement is synonymous with the  
Trp to Leu substitution involved in malathion resistance in *L. cuprina*. Both  
leucine and serine replace a bulky tryptophan residue within the active site  
and we therefore propose that this change accounts for the observed changes  
in the kinetic properties of the enzymes towards carboxylesters and OPs. In  
25 a similar manner it is believed that the substitution of the bulky Trp residue  
with other smaller residues such as Ala, Ile, Gly, Val, Thr, Cys and Met will  
have a similar effect. The finding of these similar active site mutations in  
malathion resistant strains of both *Lucilia* and *Musca* further supports our  
conclusion that these mutations are responsible for malathion resistance in  
30 these species.

**HYDROLYTIC ACTIVITY OF THE EXPRESSED PRODUCTS OF THE  
SUSCEPTIBLE AND MALATHION RESISTANT ALLELES OF *LcaE7***

35 Below we describe the activities of the expressed products of the  
susceptible and malathion resistant alleles of *LcaE7* for various

carboxylester and OP substrates. The results suggest a possible mechanism for malathion resistance in *L. cuprina* as a result of the mutation at nucleotide 752 in the *LcαE7* gene

5    a)    *In vitro* expression

The *in vitro* expression of the OP susceptible allele of *LcαE7* (clone Lc743) is described in patent application PCT/AU 95/00016 ("Enzyme based bioremediation").

10        The malathion resistant *LcαE7* full-length cDNA was cloned into the baculovirus transfer vector, Bacpac 6 (Clontech) 3' of the polyhedrin promoter. Transfections were conducted using a lipofection method with DOTAP (Boehringer Mannheim) as per King and Possee (The Baculovirus Expression System: A Laboratory Guide, Chapman & Hall, London, 1992).  
15        One  $\mu$ g of DNA of each of the resulting constructs together with 200 ng of Bacpac 6 baculovirus DNA (Clontech), linearised by digestion with the restriction enzyme BSU 36I (Promega), was incubated in a solution of HBS (hepes buffered saline) containing 15% DOTAP (Boehringer Mannheim) in a polystyrene container at room temperature for 10 minutes. The solution was  
20        then used to transfect a single well of a six well tissue culture plate pre-seeded 2 hrs previously with  $10^4$  Sf9 (*Spodoptera frugiperda*) cells in 1.5 mls Grace's medium (King and Possee, The Baculovirus Expression System: A Laboratory Guide, Chapman & Hall, London, 1992). After 12 hours, the medium was replaced with 3 mls of Grace's medium containing 10% fetal  
25        calf serum. Construct plus DOTAP, linearised virus plus DOTAP and DOTAP only controls were conducted in parallel with transfections. The transfections were harvested 4-5 days after infection and the cells isolated by centrifugation at 500g for 5 minutes. Aliquots of the resulting cell pellets were immediately stored on ice, resuspended in 10mM imidazole-HCl buffer,  
30        pH 7.0, containing 0.5% Triton X-100. Final protein concentrations in these cell extracts were between 5 and 40 mg/ml. Aliquots of the cell extracts were stored at -70°C prior to enzyme assays.

**b) Malathion hydrolysis*****Methods:***

5 MCE activity was assayed using the partition method of Ziegler, R., Whyard, S., Downe, A.E.R., Wyatt, G.R. and Walker, V.K., Pesticide Biochemistry and Physiology, 28:279 (1987) as modified by Whyard, S., Russell, R.J. and Walker V.K. Biochemical Genetics 32:9 (1994). Cell  
10 extracts were diluted 300-fold in 10mM imidazole-HCl, pH 7.0, and 150 $\mu$ l aliquots were placed in triplicate microfuge tubes. Reactions were started by the addition of 1 $\mu$ l ethanol containing [ $^{14}$ C]-malathion [Amersham; 103 mCi/mmol, 280nCi, labelled at both the methylene carbons of the succinate moiety, adjusted to 15mM (or 375 $\mu$ M - 15mM for kinetic experiments) by the  
15 addition of unlabelled malathion (99%; Riedel-de-Haën Ag., Seelze, Germany)]. The assay mixture was incubated at 25°C for 10 minutes, then 300 $\mu$ l of dilution buffer was added and the undegraded malathion extracted three times with 600 $\mu$ l of chloroform. The concentration of carboxylic acids of malathion in 300 $\mu$ l of aqueous phase was determined by liquid  
20 scintillation. Protein concentrations in the cell supernatants were determined using the Biorad Protein Assay Kit by the method of Bradford, M., Analytical Biochemistry 72:248 (1976) with bovine serum albumin as the standard. Boiled enzyme controls were performed routinely. The specific  
25 MCE activity of an extract of cells infected with non-recombinant baculovirus was at least 700-fold lower than that of cells infected with baculovirus encoding OP-susceptible or malathion-resistance alleles of *LcaE7*. This slight MCE activity that was not due to alleles of *LcaE7* was deemed a very minor source of error and subsequently ignored.

***Results:***

30

Using initial concentrations of malathion between 2.5 and 100  $\mu$ M, MCE encoded by malathion resistant and susceptible alleles of *LcaE7* exhibited a good fit to Michaelis-Menten kinetics.  $K_m$  and  $V_{max}$  were calculated for both enzymes.  $K_{cat}$  was then calculated from the  $V_{max}$  and the  
35 molarity of the *LcaE7* products in their respective cell extracts. The molarity of susceptible *LcaE7* product in the cell extract was determined by

titration with paraoxon as previously described in Newcomb, R. D., Campbell, P.M., Russell, R.J. and Oakeshott, J. G., Insect Biochemistry and Molecular Biology (in press). The molarity of the product of the malathion resistant allele of *LcaE7* was determined similarly, except that triphenylphosphate (TPP;  $1$  to  $10 \times 10^{-8}\text{M}$ ) was used instead of paraoxon. In a control experiment TPP ( $8 \times 10^{-8}\text{M}$ ) was preincubated with cell extract in triplicate for 15, 30 or 45 minutes prior to addition of the substrate malathion. There was no significant difference between the residual MCE activity at each of the preincubation times, indicating firstly that the inhibition of MCE by TPP had gone to completion and secondly, that TPP was not being turned over by MCE.

The kinetic parameters for malathion hydrolysis for the products of the malathion resistant and susceptible alleles of *LcaE7* are:

Table 5

Expressed <i>LcaE7</i> Gene Product	$K_m$ ( $\mu\text{M}$ )	$K_{cat}$ ( $\text{min}^{-1}$ )
Malathion susceptible (strain LS2)	$200 \pm 30$	$70 \pm 11$
Malathion resistant (strain RM-8)	$21 \pm 1$	$43 \pm 1$

The  $K_m$  and  $K_{cat}$  for the malathion resistant product are in reasonable agreement with those determined for the MCE enzyme purified from malathion resistant flies ( $11 \pm 0.4 \mu\text{M}$  and  $46 \pm 2$  per min, respectively;

Whyard, S. and Walker, V.K., Pesticide Biochemistry and Physiology, 50: 198, 1994).

c) Sensitivity of MCE activity to TPP

High sensitivity to inhibition by TPP is a distinctive characteristic of the MCE activity associated with malathion resistance in *M. domestica*, *C. putoria* and *L. cuprina*, consistent with potent synergism of malathion by TPP in resistant strains of these species (Shono, T., Applied and Entomological Zoology 18: 407, 1983; Bell, J.D. and Busvine, J.R., Entomology Experimental and Applied 10: 263, 1967; Townsend, M.G. and Busvine, J.R., Entomology Experimental and Applied 12: 243, 1969; Hughes, P.B., Green, P.E. and Reichmann, K.G., Journal of Economic Entomology 77: 1400, 1984; Smyth, K-A., Walker, V.K., Russell, R.J. and Oakeshott, J.G.,

Pesticide Biochemistry and Physiology, 54: 48, 1996). MCE activity encoded by the malathion resistant allele of *LcαE7* is potently inhibited by TPP, as indicated by stoichiometric inhibition of the enzyme at concentrations below  $10^{-7}$ M (see above).

5

d) α-Naphthyl acetate hydrolysis

**Methods:**

10 The initial rates of reactions between cell extracts containing the expressed products of the malathion resistant and susceptible *LcαE7* alleles and α-naphthyl acetate (α-NA) were determined at 25°C using a recording spectrophotometer and the method of Mastrapaolo and Yourno ( Analytical Biochemistry 115: 188, 1981). 6-200μM α-NA dissolved in 10μl of  
15 2-methoxyethanol was added to 0.1 M Tris-HCl pH 8.0 (980 μl) in a quartz cuvette. α-Naphthyl acetate is slowly hydrolysed in water so a background rate was recorded before starting the enzymic reaction by the addition of 10 μl of diluted cell extract. Control reactions were performed with extracts of both uninfected cells and Bacpac 6 infected cells. These controls  
20 exhibited negligible enzymic hydrolysis.

**Results:**

25 Using initial concentrations of α-NA from 6 to 200 μM, the enzymes encoded by the malathion resistant and susceptible alleles of *LcαE7* exhibited a good fit to Michaelis-Menten kinetics.  $K_m$  and  $V_{max}$  were calculated for both enzymes.  $K_{cat}$  was then calculated from the  $V_{max}$  and the molarity of the *LcαE7* products in their respective cell extracts (determined above).

30

Table 6

Expressed <i>LcαE7</i> Gene Product	$K_m$ (μM)	$K_{cat}$ (min <sup>-1</sup> )
Malathion susceptible (strain LS2)	70 ± 5	11,000 ± 300
Malathion resistant (strain RM-8)	150 ± 50	2270 ± 30

The  $K_m$  and  $K_{cat}$  for the malathion resistant product are in reasonable agreement with those determined for the MCE enzyme purified from malathion resistant flies ( $167 \pm 14 \mu M$  and 2063 per min; Whyard, S. and Walker, V.K., Pesticide Biochemistry and Physiology, 50: 198, 1994).

5

e)  $\alpha$ -Naphthyl butyrate hydrolysis

**Methods:**

10 As described above for  $\alpha$ -NA hydrolysis except that 6-200  $\mu M$   $\alpha$ -naphthyl butyrate ( $\alpha$ -NB) was used instead of  $\alpha$ -NA.

**Results:**

15 Using initial concentrations of  $\alpha$ -NB from 6 to 200  $\mu M$ , the enzymes encoded by the malathion resistant and susceptible alleles of *LcaE7* exhibited a good fit to Michaelis-Menten kinetics.  $K_m$  and  $V_{max}$  were calculated for both enzymes.  $K_{cat}$  was then calculated from the  $V_{max}$  and the molarity of the *LcaE7* products in their respective cell extracts (determined  
20 above).

Table 7

Expressed <i>LcaE7</i> Gene Product	$K_m$ ( $\mu M$ )	$K_{cat}$ ( $\text{min}^{-1}$ )
Malathion susceptible (strain LS2)	$20 \pm 5$	$18,000 \pm 2,000$
Malathion resistant (strain RM-8)	$29 \pm 4$	$9,000 \pm 400$

25 The  $K_m$  and  $K_{cat}$  for the malathion resistant product are in reasonable agreement with those determined for the MCE enzyme purified from malathion resistant flies ( $39 \pm 4 \mu M$  and 3700 per min; Whyard, S. and Walker, V.K., Pesticide Biochemistry and Physiology, 50: 198, 1994).

## f) General OP hydrolysis

In *M. domestica* there is a pattern of cross-resistance among OPs (Bell, J.D. and Busvine, J.R., Entomology Experimental and Applied 10: 263, 1967) such that parathion/diazinon resistant flies generally exhibit greater resistance factors towards OPs with two ethoxy groups attached to the phosphorus atom ('diethyl OPs') rather than two methoxy groups ('dimethyl OPs'). The converse pattern (ie greater resistance to dimethyl OPs) was observed for malathion resistant strains of *M. domestica* and *C. putoria* (Bell, J.D. and Busvine, J.R., Entomology Experimental and Applied 10: 263, 1967; Townsend, M.G. and Busvine, J.R., Entomology Experimental and Applied 12: 243, 1969). This dimethyl OP preference applies both to malathion analogues (with carboxylester groups) and general OPs (without carboxylester groups). The implication of these studies is that there is a general OP hydrolase activity intimately associated with malathion type resistance and that this OP hydrolase exhibits a preference for dimethyl OPs.

There are insufficient published data to determine whether such a dimethyl/diethyl OP cross-resistance pattern occurs in *L. cuprina*. Here we determine firstly that there is such a cross resistance pattern and secondly that the enzyme encoded by the malathion resistance allele of *LcaE7* has hydrolytic activity against OPs which lack carboxylester groups.

### **Methods:**

#### 25 i) Toxicology:

The following organophosphorus compounds were used: diazinon (O,O-diethyl O-2-isopropyl-6-methylpyrimidin-4-yl phosphorothioate, 91%, Mallinckrodt), parathion-methyl (O,O-dimethyl O-4-nitrophenyl phosphorothioate, 97.0%, Bayer), parathion (O,O-diethyl O-4-nitrophenyl phosphorothioate, 99%, Pestanal grade, Riedel-de-Haën), fenthion (O,O-dimethyl O-[3-methyl-4-(methylthio)phenyl] phosphorothioate, 98.8%, Bayer), fenthion-ethyl (O,O-diethyl O-[3-methyl-4-(methylthio)phenyl] phosphorothioate, a gift from Dr. G. Levot), dichlorvos (2,2-dichlorovinyl dimethyl phosphate, 99%, Chem Service), diethyl-dichlorvos (2,2-dichlorovinyl diethyl phosphate, a gift from Dr. J. Desmarchelier). di-

isopropyl-dichlorvos (2,2-dichlorovinyl di-isopropyl phosphate, a gift from Dr. J. Desmarchelier), malathion (S-1,2-bis(ethoxycarbonyl)ethyl O,O-dimethyl phosphorodithioate, technical grade, Nufarm), isopropyl malathion (S-1,2-bis(ethoxycarbonyl)ethyl O,O-di-isopropyl phosphorodithioate, a gift from Dr. J. Desmarchelier).

The toxicity of OPs in adult female *L. cuprina* was determined 3 or 4 days post-eclosion by application of OPs to the scutellum in 0.7  $\mu$ l dioctylphthalate (Busvine, J.R., Bell, J.D. and Guneidy, A.M., Bulletin of Entomological Research 54: 589, 1963;; Townsend, M.G. and Busvine, J.R., Entomology Experimental and Applied 12: 243, 1969). Each OP was applied to at least 20 flies at each of at least 5 different concentrations, spanning the dose causing 50% mortality (LD<sub>50</sub>). Control groups were treated with solvent but no OP. Mortality was determined 24 hours later. Data were fitted to the probit curve using the Probit Or Logit (POLO-PC) computer program (LeOra Software, 1987). This program corrects for natural mortality which was <5%. The statistic *g* ("index of significance for potency estimation") was always less than 0.5 for the 95% confidence limits of LD<sub>50</sub>.

## ii) Chlorfenvinphos hydrolysis assay:

20

Enzyme samples were diluted in 0.1 M imidazole-HCl buffer pH 7.0 ("imidazole buffer") to a final volume of 50  $\mu$ l. Reactions were started by the addition 0.5  $\mu$ l of (<sup>14</sup>C-ethyl)-chlorfenvinphos (CVP, 306.5 MBq/mmol, Internationale Isotope Munchen) from a 7.5mM stock solution in ethanol.

25 The reaction was incubated at 30°C and stopped by the addition of 300  $\mu$ l dichloromethane and 150  $\mu$ l of water followed by vigorous vortex mixing. The reactions were centrifuged to separate phases and 150  $\mu$ l of the upper, aqueous phase was taken for scintillation counting to determine the amount of <sup>14</sup>C-diethylphosphate produced by hydrolysis of CVP. Incubations with

30 boiled enzyme were also performed to control for non-enzymic hydrolysis of CVP.



**Results:****I) Toxicology:**

5 LD<sub>50</sub>s of 10 OPs were determined for the Woodside 5.2 strain  
(homozygous for a malathion resistance allele of *LcaE7*) and the Llandillo  
103 strain (homozygous for a parathion/diazinon resistance allele of *LcaE7*).  
LD<sub>50</sub>s were also determined for the OP susceptible LS2 strain in order to  
calculate resistance factors (Table 8). Woodside 5.2 flies exhibited about  
10 two- to five-fold greater resistance factors towards the dimethyl OPs,  
parathion-methyl, fenthion and dichlorvos than to their diethyl analogues,  
parathion, fenthion-ethyl and ethyl dichlorvos. Conversely, Llandillo 103  
flies exhibited about two-fold greater resistance factors towards the diethyl  
OPs, parathion and ethyl dichlorvos, than to their dimethyl analogues.  
15 parathion-methyl and dichlorvos. However, there was no significant  
difference between the resistance factors of Llandillo 103 flies for fenthion  
and fenthion-ethyl.

Among four diethyl OPs, Llandillo 103 flies have higher resistance  
factors than Woodside 5.2 flies (except fenthion-ethyl with similar resistance  
20 factors; Table 8). In contrast, Woodside 5.2 flies have higher resistance  
factors than Llandillo 103 for each of four dimethyl OPs. Thus both strains  
exhibit general OP resistance of similar potency, albeit with a bias towards  
either dimethyl or diethyl OPs.

Neither resistant strain exhibited more than 3-fold resistance to the  
25 di-isopropyl analogues of dichlorvos or malathion (Table 8).

Comparable data from *M. domestica* are available for seven of the  
test compounds. In each case resistance factors are similar in strains of the  
two species exhibiting parallel resistance types (Table 8).

Table 8 OP cross-resistance patterns in adult *L. cuprina* with comparisons to *M. domestica*.

OP Compounds <sup>1</sup>	LD <sub>50</sub> <sup>2</sup> LS2 (OP Susceptible)	LD <sub>50</sub> Llandillo 103 (Dz/para Resistant)	LD <sub>50</sub> Woodside 5.2 (Mal Resistant)	RF3 (Dz/para R)	RF (Mal R)
Diazinon (E)	57 (40-79), 3.5 ± 0.5	550 (490-630), 4.9 ± 0.7	270 (240-300), 5.1 ± 0.6	10 (20)	5 (2)
Parathion-methyl (M)	16 (11-20), 6.5 ± 0.8	185 (141-244), 3.4 ± 0.5	430 (390-490), 9.7 ± 1.5	12 (9)	27 (10)
Parathion (E)	52 (48-55), 9.2 ± 1.1	1050 (890-1280), 6.8 ± 1.2	290 (270-310), 13.3 ± 2.3	20 (35)	6 (3)
Fenthion (M)	61 (42-87), 5.2 ± 0.7	210 (180-240), 8.7 ± 1.5	320 (210-490), 3.9 ± 0.7	3 (3)	5 (7)
Fenthion-ethyl (E)	330 (290-370), 7.4 ± 1.1	690 (570-830), 8.7 ± 1.5	730 (660-870), 13.4 ± 2.6	2	2
Dichlorvos (M)	41 (35-51), 8.2 ± 1.1	150 (95-190), 5.1 ± 0.8	270 (210-340), 6.3 ± 1.0	4 (3)	7 (6)
Ethyl Dichlorvos (E)	360 (300-420), 5.0 ± 0.8	2370 (2320-2410), 54 ± 14	1100 (700-1500), 4.9 ± 1.2	7	3
Isopropyl Dichlorvos (P)	3500 (2200-4800), 4.1 ± 0.6	4600 (3400-5900), 4.1 ± 0.5	10200 (8800-12000), 8.1 ± 1.4	NS	3
Malathion (M)	550 (480-610), 6.4 ± 1.2	490 (360-600), 4.2 ± 0.9	4	NS (2)	>130 (157)
Isopropyl Malathion (P)	3600 (2700-4900), 6.3 ± 0.7	4900 (3700-6200), 8.5 ± 1.7	6400 (5900-7100), 10.1 ± 1.8	NS	1.8 (4)

1. Dimethyl (M), diethyl (E) or di-isopropyl (P) OPs.

2. LD<sub>50</sub> (ng/fly) with 95% confidence limits, slope and standard error of the probit regression line.3. Resistance Factors: ratio of the LD<sub>50</sub> of Llandillo 103 or Woodside 5.2 with the LD<sub>50</sub> of LS2. "NS" indicates an RF not significantly different from unity. Resistance factors of *M. domestica* of the appropriate resistance type are shown in parentheses (Bell, J.D. and Busvine, J.R., Entomology Experimental and Applied 10: 263, 1967; ; Townsend, M.G. and Busvine, J.R., Entomology Experimental and Applied 12: 243, 1969).

4. No mortality at this dose.

ii) Chlorfenvinphos hydrolytic activity of whole fly homogenates and expressed *LcαE7* gene products:

Whole fly homogenates of malathion resistant (strains RM-8, 60NE 1.1, 4.2, Beverly 6.2, Hampton Hill 6.1, Hampton Hill 6.2, Woodside 5.2, Rop Rmal 1, M22.2 6.3, M27.1 4.1), diazinon resistant (Gunning 107, Inverell 22, Q4, RM2.6, Llandillo 103, Sunbury 5.2) and susceptible (LBB 101, Llandillo 104, LS2) strains of *L. cuprina* were tested for esterase-mediated hydrolysis of CVP, a general OP (ie not a malathion-type OP). All 10 malathion resistant strains had greater CVP hydrolytic activities (1.5 -3.0 pmol/min/mg) than the 3 susceptible strains (0.5 -1.0 pmol/min/mg, but less activity than the 6 diazinon resistant strains (8.2 - 30.0 pmol/min/mg).

The expressed product of the malathion resistant *LcαE7* allele was tested for CVP hydrolytic activity. Turnover of 75μM CVP was about 1.2 hour<sup>-1</sup>, which is approximately 50-fold less than that of the diazinon resistant (RM2-6) *LcαE7* gene product but much greater than that of the OP-susceptible (LS2) gene product, for which CVP activity was undetectable. [The CVP hydrolytic activity of the gene products of the RM2-6 and LS2 alleles of *LcαE7* are described in patent application PCT/AU 95/00016 : "Enzyme based bioremediation"].

g) Conclusions

1. We have discovered that dimethyl versus diethyl patterns of OP cross-resistance among strains of *L. cuprina* parallel those of OP resistant strains of *M. domestica* and *C. putoria*. The two OP resistance types are equally potent and general among most OPs (excluding malathion), albeit with a dimethyl or diethyl OP preference.

2. Diethyl OP hydrolytic activities encoded by the OP susceptible allele (nil), the malathion resistant allele (1.2 hour<sup>-1</sup>) and the diazinon/parathion resistant alleles of *LcαE7* (~ 1 min<sup>-1</sup>) parallel the diethyl OP hydrolytic activities in homogenates of OP susceptible (low), malathion resistant (intermediate) and diazinon/parathion resistant (high) *L. cuprina* strains.

3. Taking points 1 and 2 together we propose that the dimethyl versus diethyl pattern of general OP cross resistance reflects the substrate specificity of the general OP hydrolase activities encoded by the two alternative OP resistance alleles of the  $\alpha E7$  gene. Thus we expect that products of malathion resistance alleles of  $\alpha E7$  genes from *L. cuprina* and *M. domestica* will exhibit dimethyl OP hydrolysis with kinetics that are as favourable for bioremediation as the diazinon/parathion resistance  $\alpha E7$  alleles are for diethyl OPs.
4. The enhanced MCE activity of the product of the malathion resistance alleles of  $\alpha E7$  genes causes flies to survive more than 100-fold greater doses of malathion. The MCE activity is enhanced in two ways. Firstly, it has more favourable kinetics for malathion breakdown than that of the susceptible allele ( $K_{cat}/K_m$  is 6-fold greater). Secondly, it has acquired general OP hydrolase activity. The latter is important for both resistance and bioremediation because it enables the enzyme to recover its MCE activity after phosphorylation/inhibition by the 'activated' or 'P=O' form of OP insecticides. P=O OPs are encountered in an insect because they are generated by an insect's metabolism. For bioremediation the OP hydrolase is required for two reasons; firstly, to hydrolyse general OPs where these are the main contaminant, and secondly, to ensure that malathion hydrolysis by the enzyme will continue in the presence of minor contamination with 'P=O' OPs.

#### ALLELISM OF THE MALATHION AND DIAZINON/PARATHION RESISTANCE PHENOTYPES IN *L. CUPRINA*

It is clear from the above molecular genetic and biochemical data that malathion resistance in *L. cuprina* is conferred by a structural mutation in the active site of *Lc $\alpha E7$*  (esterase E3), the same gene that is involved in resistance to diazinon/parathion type OPs. However, two classical genetic studies have detected a small number of presumptive recombinants between the malathion and diazinon resistance phenotypes in *L. cuprina*, which would suggest that they are separate, albeit closely linked, genes (Raftos, D.A. and Hughes, P.B., Journal of Economic Entomology 77: 553. 1986;

Smyth, K-A., Russell, R.J. and Oakeshott, J.G., *Biochemical Genetics* 32: 437, 1994).

5 The availability of frozen extracts from three of the five presumptive recombinants generated by the study of Smyth *et al.* (1994) enabled the authors to test them directly for the predicted genotypes using PCR techniques. In this particular study, diazinon resistant males (strain Q4) were crossed to malathion resistant females (strain RM-8) and the F1 females backcrossed to Q4 males. Progeny were scored for the E3-non-staining, high MCE phenotypes as indicative of resistance to both diazinon and malathion.

10 Five presumably recombinant individuals showing the MCE high/E3 non-staining phenotype were recovered from 692 backcross progeny; none were recovered with the reciprocal MCE low/E3 staining phenotype, which would presumably be susceptible to both malathion and diazinon.

#### 15 **Methods:**

The regions of the *LcσE7* gene surrounding the diazinon resistance (Gly to Asp substitution at position 137) and malathion resistance ( Trp to Leu at position 251) mutations were amplified from each of the three

20 available extracts from the Smyth *et al.* (1994) study, using the 7F1/7R2 and 7F7/7R4 primer sets, respectively. The PCR conditions and primers (except 7F7) are as described above, except that an annealing temperature of 55°C and a buffer supplied by the manufacturer of Taq DNA polymerase (BRL; 0.2 mM of each dNTP, 20 mM Tris-HCl, pH8.4, 50 mM KCl, 1.5 mM

25 MgCl<sub>2</sub>) were used. The 7F7 primer has the sequence: 5' tgctgcctctaccactacat 3' and its 5' position in the Lc743 sequence is nucleotide 660 (see Figure 1).

The 330nt fragment of *LcσE7* generated by the 7F7/7R4 primer set contains an RFLP polymorphism specific for each of the Q4 and RM-8

30 alleles: an *Nco*I cleavage site at nucleotide position 752 marks the Q4 allele (this polymorphism is at the site of the Trp to Leu mutation responsible for malathion resistance), while a *Bgl*I site at position 796 characterises the RM-8 allele. Therefore, in order to identify the Q4 and RM-8 alleles in each extract, PCR products were digested directly with each restriction enzyme

35 and the products sized by agarose gel electrophoresis, using standard techniques (Sambrook, J., Fritsch, E.F. and Maniatis, T., *Molecular Cloning*:

A Laboratory Manual, Second Edition, Cold Spring Harbor Laboratory Press, USA, 1989). Controls contained PCR products generated from Q4 and RM-8 genomic DNA.

5 No such convenient RFLP polymorphisms were contained in the  
326nt fragment amplified by the 7F1/7R2 primer set (this fragment contains  
a 68nt intron at nucleotide position 360). However, three nucleotide  
polymorphisms distinguish the Q4 and RM-8 fragments: an A to T  
substitution at nucleotide position 303, T to C at position 345 and G to A at  
10 position 410 in the Q4 sequence (the latter substitution is responsible for  
diazinon/parathion resistance). PCR products were therefore cloned into  
the pGEM-T vector (Promega) using standard techniques (Sambrook, J.,  
Fritsch, E.F. and Maniatis, T., Molecular Cloning: A Laboratory Manual,  
Second Edition, Cold Spring Harbor Laboratory Press, USA, 1989) and  
individual clones sequenced using commercially available SP6 and T7  
15 primers and dye terminator technology, as described above.

#### Results:

Digestion of the PCR products generated by the 7F7/7R4 primer set  
20 with either *Nco1* or *Bgl1* revealed the presence of only the RM-8  
chromosome in all three extracts (ie PCR products could be cleaved by *Bgl1*  
and not *Nco1*, whereas the products of the control Q4 genomic DNA were  
readily digested with *Nco1*; data not shown). Curiously, the Q4 allele was  
not amplified from any of the extracts despite the fact that F1 progeny were  
25 backcrossed to Q4 in the crossing regime.

Two out of three clones derived from the first extract, and two out of  
two clones derived from a second, contained polymorphisms characteristic  
of the Q4 allele at positions 303 and 345 and the polymorphism  
characteristic of RM-8 at position 410. On the other hand, the third clone  
30 derived from the first extract and a single clone derived from the third  
extract contained all three polymorphisms characteristic of RM-8. Again, no  
fragments generated entirely from the Q4 chromosome were found among  
the cloned DNAs.

**Conclusions:**

1. The Q4 allele was not amplified from any of the extracts despite the fact that F1 progeny were backcrossed to Q4 and would therefore be expected to contain at least one copy of the Q4 fourth chromosome.

2. Progeny homozygous for the Q4 allele would be expected if the malathion resistance mutation was located on a gene separate from *LcaE7*. No such progeny were found.

3. No MCE low/E3 staining (presumably susceptible to both malathion and diazinon) progeny were recovered, which would be expected if E3 and MCE were separate genes.

4. At least two of the extracts contained a fourth chromosome that was a Q4/RM-8 recombinant somewhere in the region of the *LcaE7* gene 5' to the Gly to Asp mutation at nucleotide position 410. The origin of flies carrying this fourth chromosome and the MCE activity / PAGE phenotype of the resultant chimeric protein are unknown.

5. It is clear that none of the putative E3/MCE recombinants were the outcome of simple reciprocal recombination events during the crossing programs; they do not, therefore, constitute proof that the E3 and MCE genes are separate genes.

It is clear from the present invention that malathion resistance in *L. cuprina* is the result of a structural mutation in the *LcaE7* (E3) gene, the same gene which mutates to give resistance to diazinon/parathion type OPs. In other words, the MCE and E3 genes are probably allelic and not separated by 0.7 map units as previous classical genetic studies had indicated. The allelism of the two resistance mutations explains the observation of Smyth, K-A., Russell, R.J. and Oakeshott, J.G. (Biochemical Genetics 32:437, 1994) that there is a negative association between malathion-type OP resistance and diazinon/parathion type OP resistance. The presence of malathion-type resistance alleles in a population would therefore suggest the use of diethyl

OPs for combating flystrike, while the presence of diazinon/parathion-type resistance alleles would suggest the use of dimethyl OPs.

5 The MCE enzyme produced by the method of the present invention may be used to develop a functional *in vitro* assay for the degradation of carboxylester and dimethyl type OPs in much the same way as the E3 enzyme (Patent: Enzyme Based Bioremediation) has been used to develop an *in vitro* assay for the general OP hydrolysis. Used together, such assays provide scope for screening for alternative OPs that might overcome resistance problems by E3-like enzymes.

10 It will be appreciated by persons skilled in the art that numerous variations and/or modifications may be made to the invention as shown in the specific embodiments without departing from the spirit or scope of the invention as broadly described. The present embodiments are, therefore, to be considered in all respects as illustrative and not restrictive.